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Synaptic Activation Causes the mRNA for the IEG *Arc* to Localize Selectively near Activated Postsynaptic Sites on Dendrites

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Summary

Polyribosomal complexes beneath postsynaptic sites on dendrites provide a substrate for local translation of particular mRNAs, but the signals that target mRNAs to synapses remain to be defined. Here, we report that high frequency activation of the perforant path projections to the dentate gyrus causes newly synthesized mRNA for the immediate-early gene (IEG) *Arc* to localize selectively in activated dendritic segments. Newly synthesized Arc protein also accumulates in the portion of the dendrite that had been synaptically activated. The targeting of *Arc* mRNA was not disrupted by locally inhibiting protein synthesis, indicating that the signals for mRNA localization reside in the mRNA itself. This novel mechanism through which newly synthesized mRNA is precisely targeted to activated synapses is well suited to play a role in the enduring forms of activity-dependent synaptic modification that require protein synthesis.

Introduction

Long lasting forms of activity-dependent synaptic modification are thought to require gene expression (Kang and Schumann, 1996; Nguyen and Kandel, 1996; Frey and Morris, 1997; Martin et al., 1997), but the events between synaptic activation, gene induction, and the modification of individual synapses remain to be defined. The characteristics of enduring forms of long-term potentiation (LTP) imply that the mechanism probably involves (1) a signal transduction event at the activated synapses that marks them for subsequent modification (Frey and Morris, 1997), (2) a signaling process that modulates gene expression in the postsynaptic neuron, (3) the synthesis of particular gene products that are necessary for bringing about the synaptic modifications, and (4) the delivery of these gene products to the individual synapses that are to be modified. All of these events must be coordinated in such a way that modifications occur selectively in those synapses that experienced the appropriate patterns of activity.

One mechanism that could mediate several of the required functions simultaneously involves synapse-specific gene expression, whereby particular mRNAs are translated locally at postsynaptic sites on dendrites. The idea of synapse-specific gene expression had its roots in the discovery of "synapse-associated polyribosome complexes" (SPRCs)—polyribosomes and associated membranous cisterns that are selectively localized beneath postsynaptic sites on the dendrites of CNS neurons (Steward and Levy, 1982; Steward, 1983; Steward and Fass, 1983). The selective localization of SPRCs beneath synapses implied that signals generated by the synapse somehow caused SPRCs to localize selectively in the postsynaptic cytoplasm.

Since the discovery of SPRCs, several of the mRNAs that are present in dendrites have been identified (Steward, 1997; Steward and Singer, 1997). However, two fundamental questions have remained unanswered: (1) whether certain mRNAs are delivered selectively to postsynaptic sites so as to mediate synapse-specific gene expression, and (2) whether signaling events at individual synapses regulate mRNA localization. The present study provides new insights into these questions by demonstrating for the first time that appropriate patterns of synaptic activity cause one newly synthesized mRNA to localize selectively within activated dendritic domains.

Our experiments to identify the signals that regulate the intracellular trafficking of newly synthesized mRNA have focused on the transcript of an immediate-early gene (IEG) named *Arc*, for activity-regulated cytoskeleton-associated protein (Lyford et al., 1995), also known as Arg3.1 (Link et al., 1995). *Arc* offers special advantages for studies of mRNA trafficking, because it is expressed as an IEG, yet its mRNA is rapidly delivered into dendrites (Lyford et al., 1995; Wallace et al., 1998). Hence, the intracellular trafficking of the mRNA can be studied in a way that is not possible with mRNAs that are expressed constitutively.

To determine if signaling events at synapses regulate the localization of *Arc* mRNA within dendrites, we took advantage of the projection from the medial entorhinal cortex (EC) to the dentate gyrus, the medial perforant path (MPP), which terminates in a precisely defined lamina in the middle molecular layer of the dentate gyrus (Steward, 1976). This pathway can be selectively activated by positioning stimulating electrodes in the medial EC, so as to produce a band of activated synapses in the molecular layer. This is the pathway in which LTP was first described (Bliss and Lomo, 1973), and the late phase of this form of LTP requires transcription and translation (Krug et al., 1984; Otani and Abraham, 1989; Otani et al., 1989; Nguyen and Kandel, 1996).

Here, we demonstrate that high frequency activation of perforant path synapses both induces *Arc* expression and causes newly synthesized *Arc* mRNA to localize selectively in the synaptically activated dendritic lamina. The exact localization pattern of *Arc* mRNA depends on the synapses that were activated. Newly synthesized Arc protein also accumulates in the activated dendritic lamina, suggesting that localization of the mRNA underlies a local synthesis of Arc protein. The distribution of

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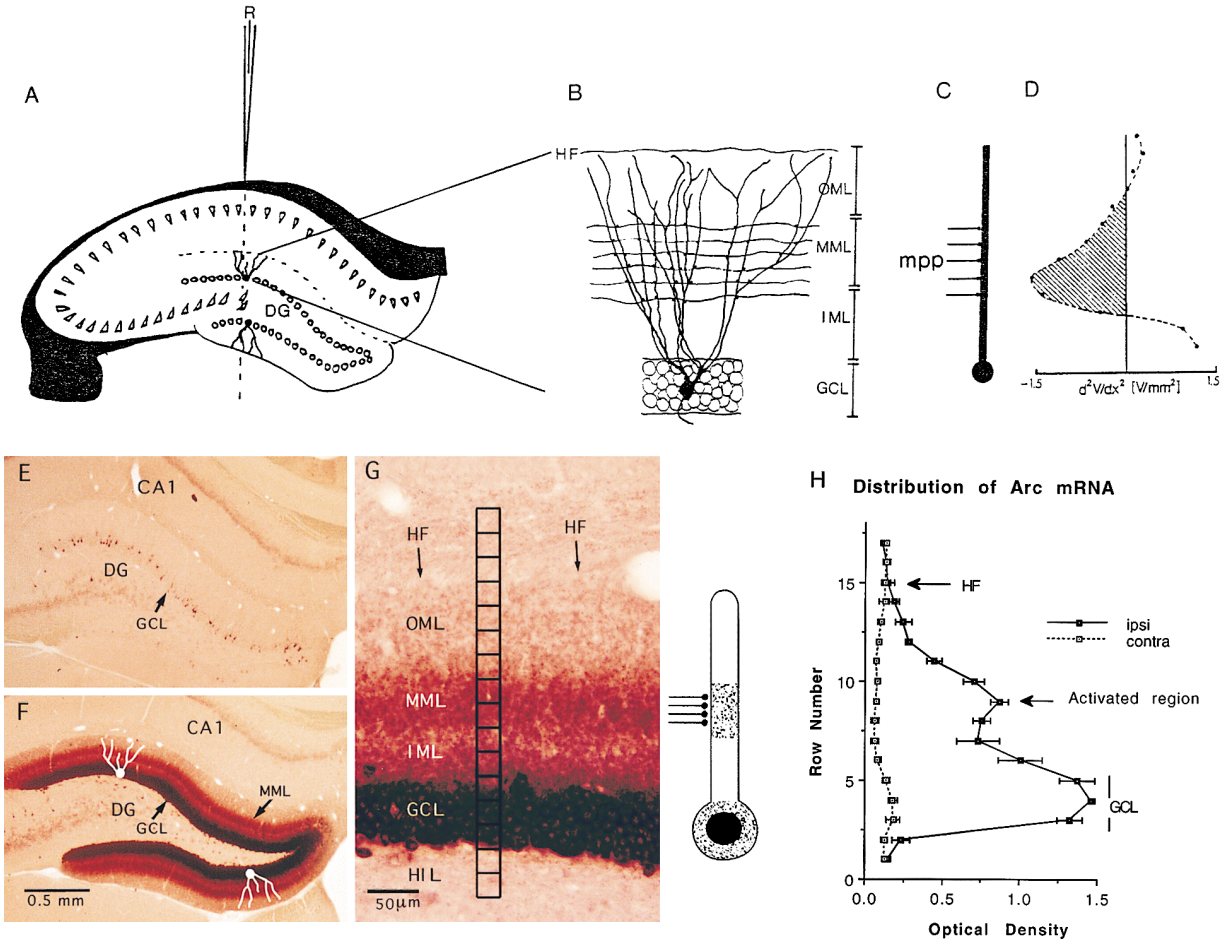

Figure 1. Repeated High Frequency Stimulation of the Pathway from the Medial EC to the Dentate Gyrus Leads to a Selective Localization of Recently Synthesized *Arc* mRNA in Activated Dendritic Domains

(A) Overall organization of the hippocampal formation and the path of the recording electrode.
(B) Schematic illustration of how axons from the medial EC terminate on dentate granule cells.
(C) Simplified cartoon illustrating the pattern of termination of synapses of the MPP on granule cell dendrites.
(D) Current source density analysis of the depolarizing synaptic currents generated by stimulation of the MPP fibers.
(E and F) Distribution of *Arc* mRNA as revealed by digoxygenin-labeled cRNA probes in nonactivated dentate gyrus (this is the contralateral side of the animal illustrated in (F) and after delivering high frequency trains to the MPP over a 2 hr period.
(F) The drawings of neurons (in white) illustrate the location of granule bodies and the orientation of dendrites in the dorsal and ventral blades. Note the prominent band of labeling in the middle molecular layer, which contains the middle segments of granule cell dendrites.
(G) High magnification view of the distribution of *Arc* mRNA after 2 hr of perforant path stimulation (this is a different animal than the one illustrated in [F]). The stick figure to the right schematizes the intracellular distribution of *Arc* mRNA.
(H) Graph illustrating the average OD of labeling across the molecular layer from the case illustrated in (F) and on the contralateral (control) side of the same section. Bars indicate the standard deviation of the five measurements at each level.
Abbreviations: HF, hippocampal fissure; OML, outer molecular layer; MML, middle molecular layer; IML, inner molecular layer; GCL, granule cell layer; mpp, medial perforant path. (D) is from White et al. (1990).

other constitutively expressed mRNAs is not affected. Finally, we demonstrate that the localization of *Arc* mRNA is not disrupted by inhibiting protein synthesis, indicating that localization depends on a signal in the mRNA itself.

Results

The experimental setup is schematically illustrated in Figure 1. The perforant path projections from the EC to the dentate gyrus terminate in a topographically organized fashion along the dendrites of dentate granule cells. In its entirety, the perforant path innervates the outer two-thirds of the molecular layer of the dentate gyrus. Within that zone, the medial EC projects to the middle molecular layer, while the lateral EC projects to the outer molecular layer (see Figure 1B, which schematically illustrates the pattern of termination of the projections from the medial EC). These are not two entirely distinct pathways; instead, the projections are topographically organized in a gradient along the dendrite. Hence, by positioning a stimulating electrode in the medial EC, it is possible to selectively activate a band of synapses that terminate on mid-proximodistal dendrites as schematically illustrated in Figure 1C. This is demonstrated directly in Figure 1D using current source density

analysis. This graph illustrates that following activation of the MPP, inward current flow (indicative of synaptic depolarization) occurs in a discrete lamina in the middle portion of the molecular layer (from White et al., 1990).

In initial experiments, the MPP was activated by using the stimulation paradigm that is typically used to induce LTP in the dentate gyrus (400 Hz trains, 8 pulses per train, delivered at a rate of 1/10 sec). Trains were delivered repeatedly over a 2 hr period at an intensity that initially evoked an approximately half-maximal population spike (1–3 mV amplitude). Recordings carried out before and after the stimulation period indicated that the stimulation produced robust synaptic potentiation in every experiment (data not shown).

Arc mRNA Is Differentially Targeted to Dendritic Domains that Have Been Synaptically Activated

Previous studies have shown that brief periods of high frequency stimulation of the perforant path strongly induce *Arc* expression in dentate granule cells and that the newly synthesized *Arc* mRNA migrates into dendrites (Lyford et al., 1995). After the longer stimulation periods used here, there was a strikingly different labeling pattern; in addition to the high levels of labeling in granule cell bodies, there was a striking band of labeling for newly synthesized *Arc* mRNA in the middle molecular layer that corresponded exactly to the location of the band of synapses that had been activated (compare Figure 1E, which illustrates the resting levels of *Arc* mRNA in the nonactivated dentate gyrus contralateral to the stimulation, with Figure 1F, which illustrates the distribution of *Arc* mRNA following 2 hr of stimulation of the MPP). High magnification views of a different case stimulated in an identical fashion (Figure 1G) reveal a diffuse granular pattern of labeling in the molecular layer, with no indication of labeling in glial cell bodies that are present throughout the molecular layer. These facts, and the fact that the labeling following stimulation is first seen in granule cell bodies (Figure 2), strongly support the interpretation that the mRNA that accumulates in the molecular layer is in the dendrites of granule cells.

The degree of enrichment of labeling in the activated lamina was assessed by measuring the optical density (OD) of the labeling across the molecular layer. As illustrated in Figure 1H, there was a distinct peak of labeling in the middle portion of the molecular layer, indicating an accumulation of the newly synthesized *Arc* mRNA in the middle dendritic regions. A distinct band of labeling for the newly synthesized *Arc* mRNA was seen in every experiment in which the MPP was activated in this fashion (nine out of nine experiments); three different cases are illustrated in Figures 1E, 1G, and 2C to illustrate the consistency of the findings. Quantitative analyses of OD across the molecular layer in five representative cases that experienced 2 hr of stimulation of the MPP indicated that the peak OD in the middle molecular layer was an average of 18% higher than the OD in the inner molecular layer (which contains proximal dendrites) and 283% higher than the OD in the outer molecular layer (which contains distal dendrites). These results suggest that the *Arc* mRNA that had been synthesized in granule cell nuclei passed through the proximal dendritic zones and accumulated in the middle dendritic regions that had been selectively activated. It should be emphasized that *Arc* is unique among known IEG mRNAs; there is no detectable labeling for other IEG mRNAs in any portion of the dendritic laminae after induction (see below and Lyford et al., 1995; Wallace et al., 1998).

To evaluate the time course of the localization process, we compared the distribution of *Arc* mRNA after 30 min, 1 hr, and 2 hr of high frequency stimulation of the MPP. After 30 min, *Arc* mRNA was strongly induced in dentate granule cells, but there was no labeling in the molecular layer (compare Figure 2A, control, with Figure 2B). The complete absence of labeling in the molecular layer after 30 min again emphasizes that the mRNA is not expressed by glial cells. After 1 hr of stimulation, there was a clear band of labeling in the middle molecular layer (Figure 2C). Indeed, the pattern of labeling at this time appeared qualitatively similar to the pattern at 2 hr (Figure 2D). Hence, *Arc* mRNA appears to localize selectively in the synaptically activated lamina as it migrates into the dendrites. Figure 2E illustrates a quantitative assessment of the average OD levels across the granule cell layer and molecular layer in representative cases at the different time points.

The Pattern of Localization of *Arc* mRNA Depends on the Afferents that Are Activated

Different afferent projections to the dentate gyrus terminate in different laminae in the molecular layer. Hence, it was of interest to determine whether *Arc* mRNA could be differentially targeted by activating synapses that terminate at various proximodistal levels on the dendrite. Figure 3 illustrates this experiment. In the case illustrated in Figure 3A, the commissural projection to the dentate gyrus was stimulated at high frequency for 2 hr. The synapses of the commissural pathway are excitatory and terminate in the inner third of the molecular layer. However, stimulation of this pathway also evokes strong GABAergic inhibition; indeed, the induction of LTP in this pathway requires that this inhibition be blocked (Steward et al., 1990). Hence, for this experiment, we used the same strategy previously used to permit LTP, whereby the recording micropipette contained 40 mM bicuculline. We have previously shown that the diffusion of the bicuculline from the pipette blocks GABAergic inhibition locally in an area of about 1 mm in diameter (Steward et al., 1990). As illustrated in Figure 3A, stimulation of the commissural pathway strongly induced *Arc* expression in a small region near the recording electrode. In the area in which *Arc* was induced, there was a highly defined band of labeling for newly synthesized *Arc* mRNA in the inner molecular layer—exactly the layer in which the commissural pathway terminates. Note the sharp distal boundary of labeling, representing the sharp boundary between the area of the molecular layer innervated by the commissural pathway and the middle molecular layer, which is innervated EC. The graph in Figure 3F illustrates a plot of OD across the granule cell layer and molecular layer in the case illustrated in 3A. For comparison, Figures 3B and 3G illustrate the selective localization of *Arc* mRNA in the middle molecular layer following activation of the

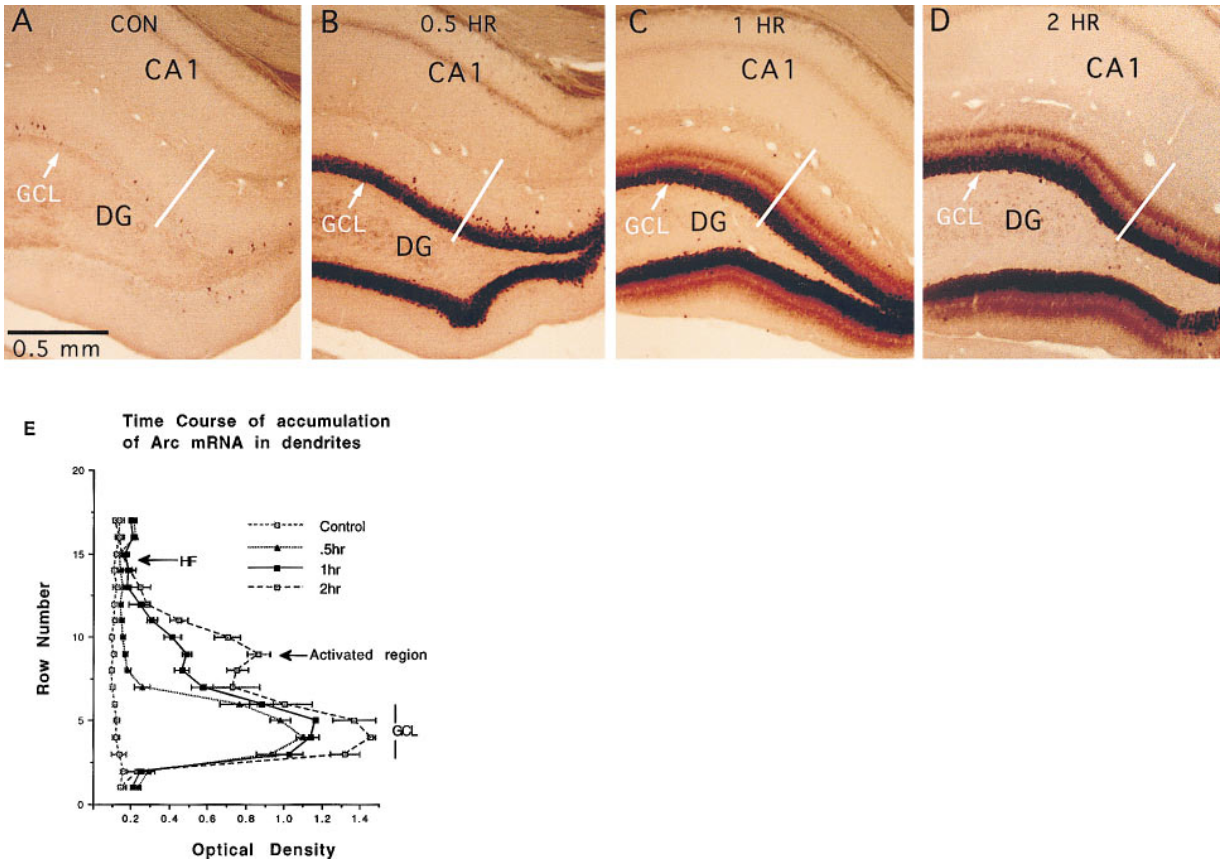

Figure 2. Time Course of Accumulation of *Arc* mRNA in Activated Dendritic Laminae

(A) through (C) illustrate the distribution of *Arc* mRNA in the nonstimulated dentate gyrus (A) and after 30 min (B), 1 hr (C), and 2 hr (D) of high frequency activation of the MPP. The white bars illustrate the approximate sites from which the OD measurements were taken.

(E) Average OD levels across the granule cell layer and molecular layer in representative cases at the different time points. Abbreviations are the same as in Figure 1.

MPP. Figures 3C and 3H illustrate the pattern of labeling following activation of projections from the lateral EC that terminate on more distal dendritic segments. In the case illustrated in Figure 3C, the overall extent of *Arc* induction was somewhat less (consistent with the fact that the more distally terminating synapses are less capable of producing strong depolarization at the cell body than those terminating more proximally). Nevertheless, there was a clearly discernable band of labeling in distal dendritic regions (Figures 3C and 3H). Thus far, we have been unable to target *Arc* mRNA to the most distal dendritic regions innervated by the lateralmost portion of the EC. This is likely due to the fact that these projections are not sufficiently powerful to strongly depolarize the distal dendrites.

The lamina-specific patterns of localization following afferent activation are distinctly different than the uniform distribution of the induced mRNA throughout the dendritic laminae that is seen after a seizure (Figures 3D and 3I). For example, Figure 3D illustrates the pattern of labeling in a case in which a seizure occurred as a consequence of the penetration of a recording electrode into the hippocampus. Such seizures occur occasionally during electrode placement. Usually, when such seizures occur, the experiment is terminated, because we have previously found that such seizures induce *Arc* expression in the dentate gyrus. In this experiment, however, the animal was allowed to survive for 2 hr and was then prepared for in situ hybridization. This pattern of labeling, in which *Arc* mRNA is uniformly distributed across the molecular layer, is identical to what is seen following electroconvulsive seizures (see below) and is distinctly different than what is seen following selective activation of particular afferents.

The Events that Trigger *Arc* Expression versus Localization Are Separable

Both synaptic activation and seizures induce *Arc* expression, but only the former leads to the selective localization of *Arc* mRNA in particular dendritic laminae. This raises the possibility that induction and localization are regulated separately. Hence, additional experiments were carried out to further evaluate the relationship between synaptic activation, the induction of *Arc* expression, and the targeting of the mRNA to active synaptic sites.

It has previously been shown that low frequency activation of the perforant path in unanesthetized animals does not induce *Arc* expression in dentate granule cells, whereas high frequency stimulation is effective (Lyford

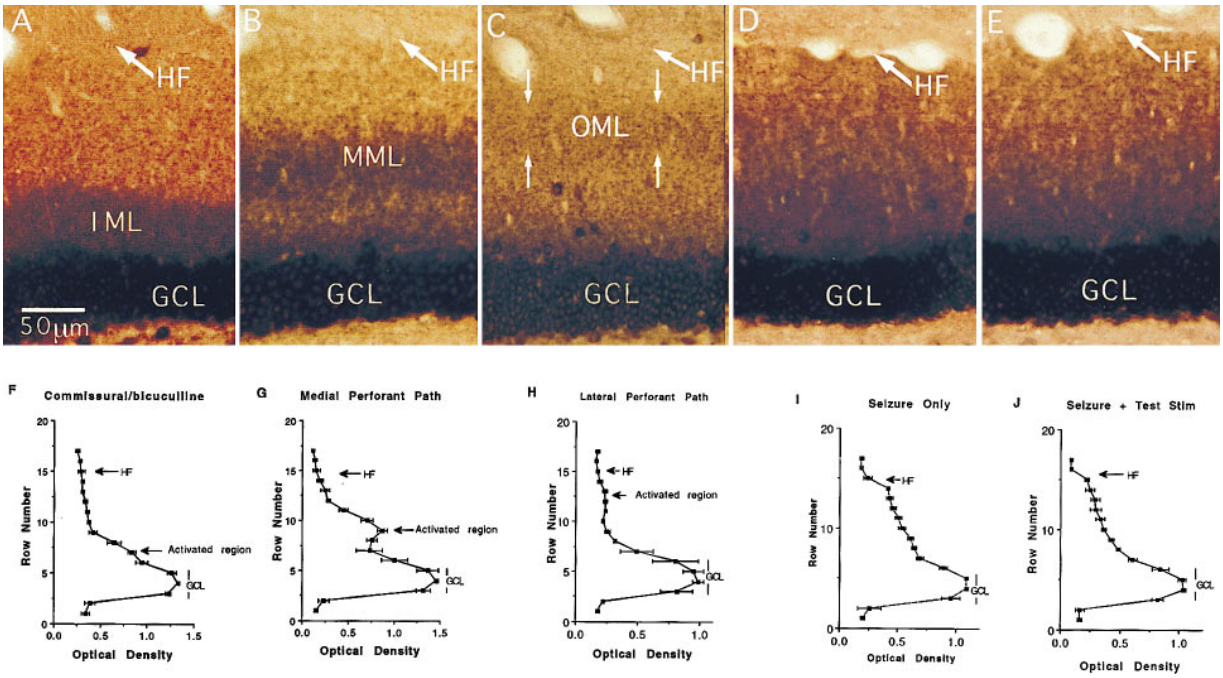

Figure 3. The Pattern of Localization of *Arc* mRNA Depends on the Afferents that Are Activated

(A) Distribution of *Arc* mRNA after high frequency activation of the commissural projection to the dentate gyrus, which terminates in the inner third of the molecular layer. In this experiment, the recording micropipette contained 40 mM bicuculline in order to block GABAergic inhibition locally. (F) illustrates the corresponding plot of OD across the granule cell layer and molecular layer.

(B) Distribution of *Arc* mRNA after activation of the MPP. (G) illustrates the corresponding plot of OD across the granule cell layer and molecular layer.

(C) Distribution of *Arc* mRNA after activation of projections from the lateral EC, which terminate on more distal dendritic segments. (H) illustrates the corresponding plot of OD across the granule cell layer and molecular layer.

(D) Distribution of *Arc* mRNA 2 hr after a seizure triggered inadvertantly by the penetration of a recording electrode into the hippocampus. (I) illustrates the corresponding plot of OD across the granule cell layer and molecular layer.

(E) Distribution of *Arc* mRNA after a seizure triggered inadvertantly by the penetration of a recording electrode into the hippocampus, which was then followed by 2 hr of test stimulation at low frequency (0.8 pulses/sec). (J) illustrates the corresponding plot of OD across the granule cell layer and molecular layer.

et al., 1995). To confirm that the same is true under the present experimental conditions, in which stimulation was delivered during acute neurophysiological experiments in anesthetized animals, we delivered the same number and intensity of stimuli over the same time period (2 hr) as in the high frequency stimulation paradigm but at a lower frequency that does not cause LTP (0.8 pulses/sec). Stimulation at this frequency led to depression of the evoked responses rather than potentiation and produced neither an induction of *Arc* expression nor any indication of mRNA accumulation in the middle molecular layer of the dentate gyrus (data not shown).

Because *Arc* was not induced by the low frequency stimulation, it could not be determined whether the stimulation would be sufficient for localization if the mRNA was present. To evaluate this possibility, we again took advantage of the fact that the penetration of a recording electrode into the dentate gyrus occasionally triggers a seizure. In an experiment in which a seizure occurred, we delivered test stimulation as the response recovered (~3 min), set the stimulus intensity so as to evoke a population spike of ~3 mV, and then continued test stimulation at a rate of 1/10 sec for 2 hr. As illustrated in Figure 3E, the low frequency stimulation did not cause *Arc* mRNA to localize within the activated laminae; instead, the mRNA that had been induced by the seizure was distributed uniformly across the molecular layer in a pattern that was identical to what was seen following a seizure alone (compare with Figure 3D). The graphs in Figures 3F–3J illustrate the levels of *Arc* mRNA across the granule cell layer and molecular layer in the cases illustrated in Figures 3A–3E.

Next, we evaluated whether newly synthesized *Arc* mRNA that was induced by a generalized seizure could be targeted selectively as a consequence of subsequent synaptic activation. For this experiment, *Arc* expression was induced bilaterally by eliciting a single electroconvulsive seizure (ECS) in an animal that had been anesthetized and prepared for acute neurophysiological recording. Then, beginning 10 min after the ECS, high frequency stimulation was delivered to the MPP in one hemisphere for 2 hr. On the side on which the perforant path had been activated after the ECS, newly synthesized *Arc* mRNA was localized selectively in the activated dendritic domain (Figure 4A). In contrast, *Arc* mRNA was uniformly distributed in the molecular layer on the ECS-only side (Figure 4B).

It was noteworthy that the levels of labeling in the outer molecular layer on the stimulated side were lower than on the ECS-only side. This labeling pattern suggests that the accumulation of the newly synthesized mRNA in the dendritic domains that had been strongly

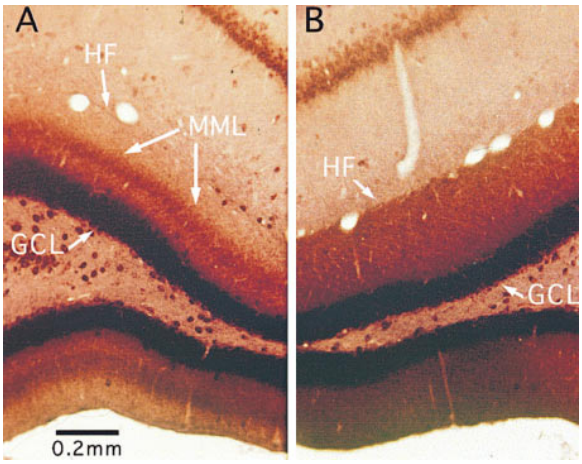

Figure 4. The Induction and Targeting of *Arc* mRNA Are Independently Regulated

A single ECS was elicited by delivering a 40 mA/60 Hz current (0.5 sec duration) via ear clip electrodes in an animal that had been anesthetized with Nembutal and prepared as described above for stimulation and recording. Then, high frequency trains were delivered over a 2 hr period to the MPP on one side.

(A) Distribution of *Arc* mRNA on the side on which afferent stimulation had been delivered after inducing a single ECS.

(B) Distribution of *Arc* mRNA on the side that did not receive MPP stimulation. Note the selective localization of *Arc* mRNA on the stimulated side and the uniform distribution on the ECS-only side. Abbreviations are the same as in Figure 1.

activated (the middle molecular layer) may have prevented the mRNA from migrating further into distal dendritic domains (the outer molecular layer). It is also possible, however, that synaptic activation of middle dendritic regions somehow accelerated mRNA degradation in distal dendritic zones.

Taken together, these results indicate that low frequency stimulation is not sufficient to induce the selective targeting of *Arc* mRNA; in contrast, high frequency trains that are sufficient to induce LTP are effective. The results also suggest that the mRNA induced by a generalized seizure can be targeted by signals that are generated subsequently via synaptic activation. It cannot be completely excluded, however, that the afferent stimulation causes a degradation of the mRNA that had been induced by the seizure and that only the mRNA that was synthesized in response to the localized synaptic activation was targeted to the activated laminae.

Localization of *Arc* mRNA in Activated Dendritic Laminae Is Associated with a Local Accumulation of Arc Protein

Immunostaining of tissue sections from stimulated animals using an Arc-specific antibody revealed a clear band of newly synthesized protein in the same dendritic laminae in which *Arc* mRNA was concentrated (compare Figure 5A, control, with Figure 5B, stimulated). A discrete band of immunostaining was also seen in the animal that received a single ECS followed by perforant path stimulation (Figure 5C). No such band was seen on the ECS-only side (Figure 5D), consistent with previous

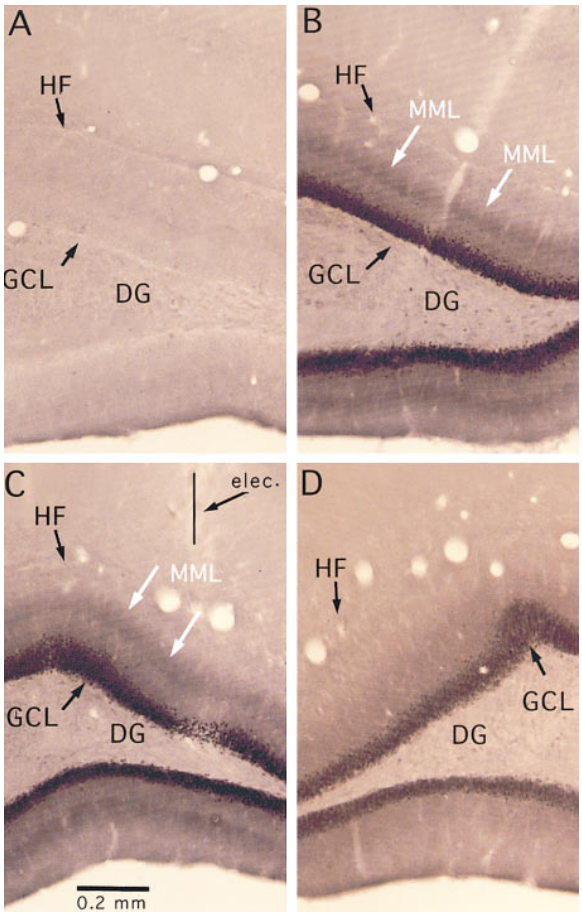

Figure 5. Localization of *Arc* mRNA in Activated Dendritic Laminae Is Associated with a Local Accumulation of Arc Protein

(A) and (B) illustrate the immunostaining pattern for Arc protein after 2 hr of high frequency stimulation of the perforant path ([A], nonactivated side; [B], synaptically activated side).

(C and D) Immunostaining pattern for Arc protein in the animal that received a single ECS and then high frequency stimulation of the perforant path (compare with Figure 4). The track of the recording electrode can be seen on the right hand side of the photograph in (C).

Abbreviations are the same as in Figure 1.

studies indicating a uniform distribution of immunostaining following ECS (Lyford et al., 1995; Wallace et al., 1998).

The fact that synaptic activation leads to the selective targeting of both recently synthesized mRNA and protein suggests that the targeting of the mRNA underlies a local synthesis of the protein. An alternative possibility, however, is that mRNA localization is actually mediated by the selective targeting of the nascent protein as it is being translated. To exclude the latter possibility, we assessed whether mRNA localization was disrupted when protein synthesis was blocked. We inhibited protein synthesis locally by positioning micropipettes filled with puromycin (25 mg/ml in saline) or cycloheximide (20 mg/ml in saline) in the dentate gyrus; the inhibitor-filled micropipettes also served as a recording electrode. Both puromycin and cycloheximide were used because of their different modes of action. Cycloheximide inhibits protein synthesis by blocking elongation

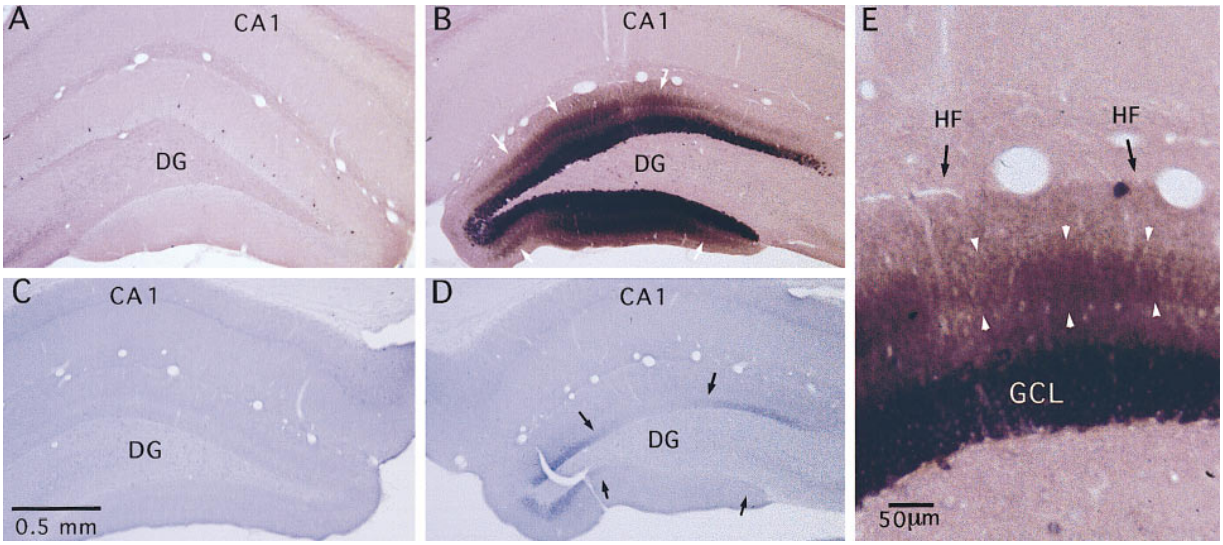

Figure 6. The Selective Localization of Arc mRNA in Activated Dendritic Domains Is Not Blocked by Inhibiting Protein Synthesis

The photomicrographs illustrate the distribution of *Arc* mRNA as revealed by digoxygenin-labeled probes.
(A) Nonstimulated (control) side.
(B) Stimulated side (2 hr of stimulation as described in Figure 1).
A recording micropipette filled with puromycin (25 mg/ml in saline) was present on the stimulated side. The track of the micropipette can be seen in (B), and the very small area of decreased labeling indicates the area that was directly damaged by the pipette. (C) and (D) illustrate the immunostaining pattern for Arc protein in nearby sections. The diffusion of the puromycin from the pipette produced an area several hundred micrometers in diameter in which protein synthesis was inhibited as documented by the absence of induced expression of Arc protein (see arrows in [D]). Note the blockade of Arc protein expression in part of the dentate gyrus (see arrows in [D]), indicating the area of effective protein synthesis inhibition. A laminated pattern of labeling for Arc mRNA is still evident as documented by the high magnification photomicrograph in (E), which is taken in the center of the area of inhibition. Note the band of increased labeling in the middle molecular layer (white arrowheads) and the relatively higher levels of labeling for *Arc* mRNA in the areas in which protein synthesis had been inhibited. Abbreviations are the same as in Figure 1.

of the nascent peptide chain; puromycin is incorporated into the peptide chain, causing premature peptide chain termination and disaggregating mRNA and ribosomes (Reid et al., 1970). High frequency stimulation was delivered, and the localization of *Arc* mRNA was evaluated in the area in which protein synthesis was blocked. The area of effective protein synthesis inhibition was defined by immunostaining sections for Arc protein or for c-fos protein, another IEG that is induced by high frequency stimulation. Areas of effective inhibition were identified by the absence of induced Arc or c-fos protein (for an example of immunostaining for Arc protein, see Figure 6D).

As illustrated in Figures 6B and 6E, *Arc* mRNA still accumulated selectively in the middle molecular layer in the areas in which protein synthesis was inhibited by puromycin, even though the levels of *Arc* mRNA were higher in the area of protein synthesis inhibition (Figure 6). Similar results were obtained with cycloheximide-filled micropipettes. Activity-dependent localization of *Arc* mRNA was also unaffected in two animals that received systemic injections of cycloheximide (20 mg/kg intraperitoneally, data not shown). The fact that *Arc* mRNA was selectively localized when protein synthesis is inhibited indicates that localization of the mRNA is not determined by the encoded protein. Although it cannot be completely excluded that there are separate mechanisms for targeting the mRNA and protein, the most direct explanation is that the targeting of the mRNA underlies a local synthesis of Arc protein in the activated dendritic laminae.

As an aside, neither puromycin nor cycloheximide blocked the synaptic potentiation that was seen immediately after high frequency stimulation (data not shown). This is expected, since treatment with protein synthesis inhibitors block only the late phase of perforant path LTP; the potentiation that is seen immediately after high frequency stimulation is not affected (Frey et al., 1988).

Synaptic Activation Does Not Cause Other IEG mRNAs to Localize in Dendrites or Alter the Distribution of mRNAs that Are Present Constitutively in Dendrites

One explanation for the selective localization of newly synthesized *Arc* mRNA to the activated dendritic laminae is that synaptic activation generates some "tag" that causes an mRNA with an appropriate localization signal to dock selectively. Alternative possibilities are that (1) synaptic activation causes a general reorganization of the dendritic cytoplasm or cytoskeleton, such that any mRNA would be trapped in the synaptically activated laminae, or (2) activation of granule cells via one set of synapses caused a selective degradation of mRNA in nonactivated dendritic laminae. To explore these alternative possibilities, we evaluated whether perforant path stimulation led to the accumulation of the mRNA for another IEG in the dendritic laminae (the mRNA for NGFI-A (Milbrandt, 1987), also called *Zif 268*

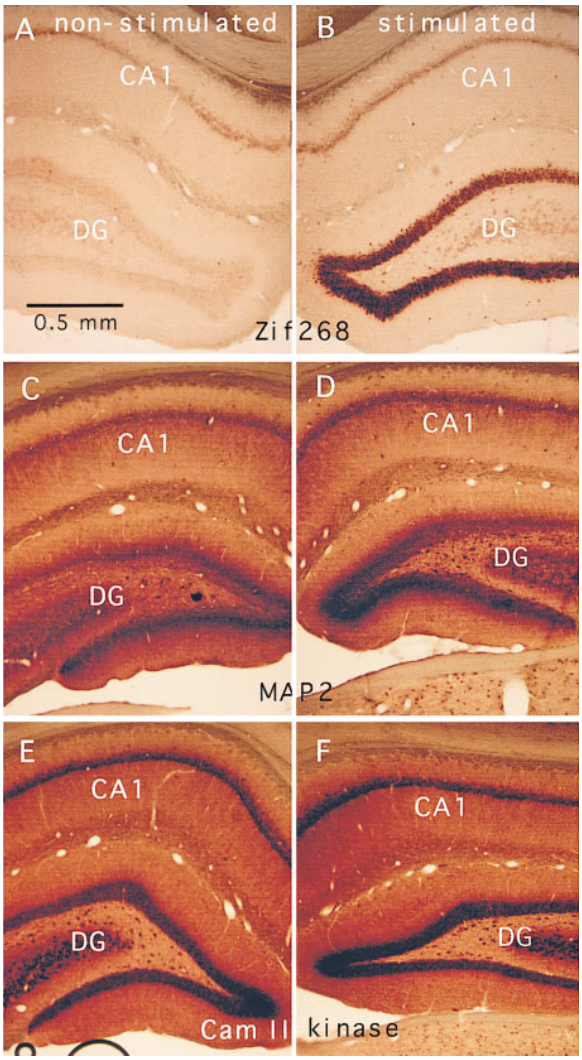

Figure 7. Synaptic Activation Does Not Cause Other IEG mRNAs to Localize in Dendrites or Alter the Distribution of mRNAs that Are Present Constitutively in Dendrites

These panels illustrate the distribution of the mRNAs for *Zif 268*, MAP2, and CaM Kinase II in an animal that had received high frequency stimulation of the MPP for 2 hr.
(A and B) Distribution of the mRNA for *Zif 268*.
(C and D) Distribution of the mRNA for MAP2.
(E and F) Distribution of the mRNA for CaM Kinase II.
Nonstimulated side, (A), (C), and (F); stimulated side, (B), (D), and (F). Abbreviations are the same as in Figure 1.

(Christy et al., 1988). NGFI-A/*Zif 268* is strongly induced by the same patterns of activity that induce *Arc* but normally remains tightly localized to the cell body region. We also evaluated whether afferent activation altered the distribution of two other mRNAs that are present constitutively in dendrites (the mRNAs for CaM Kinase II and MAP2). Figures 7A and 7B illustrate that *Zif 268* mRNA was strongly induced following 2 hr of stimulation, but labeling remained tightly localized to the granule cell body layer; there was no evidence of labeling within the activated dendritic laminae. Moreover, there was minimal if any alteration in the pattern of labeling for the mRNAs for CaM Kinase II (compare Figures 7B and 7C) or MAP2 (Figures 7D and 7E) following 2 hr of high frequency stimulation of the perforant path. These results indicate that the selective localization of newly synthesized *Arc* mRNA cannot be due to some general alteration in the dendritic cytoskeleton or to a general degradation of mRNA in the nonactive laminae.

Discussion

The present study reveals the existence of a previously unknown mechanism through which newly synthesized mRNA is targeted to dendritic regions that have been activated by afferent stimulation. These results add to the emerging story that mRNA trafficking within neurons is a regulated process (Knowles and Kosik, 1997) and demonstrate that synaptic activity creates some signal that causes certain mRNAs to localize at or near the active postsynaptic sites. These findings indicate that neurons possess a mechanism that permits them to selectively address newly synthesized mRNA to synapses based on their activational history. It is not yet clear how precise this localization mechanism is; in particular, it remains to be seen whether the mRNA is selectively localized at the active synapses or is more generally localized in the region of the dendrite contacted by the active synapses. In either case, the mechanism that underlies the targeting of newly synthesized mRNA to activated dendritic domains is well suited to play a role in the long lasting forms of activity-dependent synaptic modification that require protein synthesis.

The Expression Dynamics of *Arc* Provide Unique Advantages for Studies of mRNA Trafficking

Arc mRNA offers particular advantages for studies of mRNA trafficking within neurons, because it is expressed as an IEG. *Arc* mRNA is expressed at very low levels constitutively but is strongly induced by neuronal activity; hence, the synthesis, intracellular transport, localization, and life history of *Arc* mRNA can be studied in a way that is not possible with mRNAs that are expressed constitutively. Previous studies of *Arc* have provided insights into the dynamics of mRNA transport in dendrites and the targeting signals that select certain mRNAs from a vast pool of other transcripts for export into dendrites (Wallace et al., 1998). The present results extend the story by demonstrating that not only the routing into dendrites but also the intradendritic localization of the newly synthesized mRNA are precisely controlled. It remains to be seen whether *Arc* mRNA has features that are entirely unique or whether *Arc* is a representative of a group of mRNAs that are targeted in a similar way.

mRNA Trafficking in Neurons Is a Multistep Process

Studies of other cell types in which mRNAs are differentially localized suggest that mRNA trafficking involves two distinct steps: transport from one site to another followed by a docking process that mediates selective localization (Yisraeli et al., 1990; Steward and Singer, 1997). For example, in oligodendrocytes, separate domains within myelin basic protein mRNA determine transport into processes versus localization in membrane sheets (Ainger et al., 1997). Our data suggest that the trafficking of *Arc* mRNA in neurons is also a two

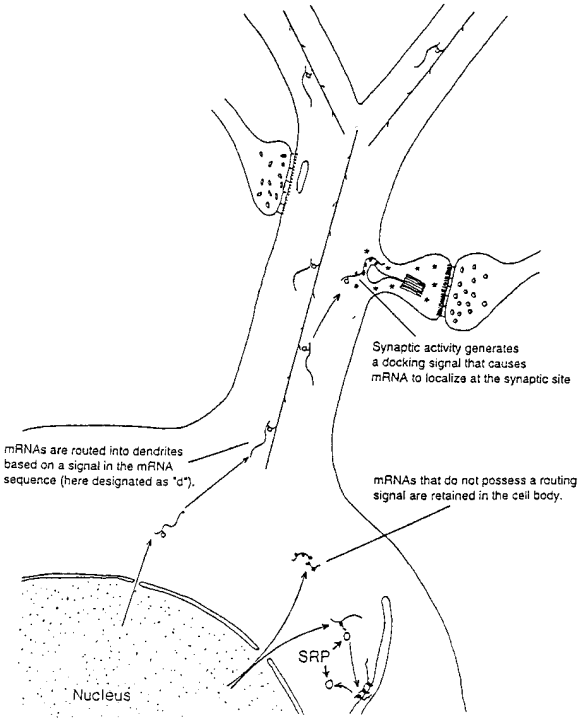

Figure 8. The Steps Involved in Delivering mRNA to Synapses

step process and that each step can be regulated independently. For example, postsynaptic activity induces *Arc* mRNA synthesis and leads to the delivery of the mRNA throughout dendrites. Synaptic activation then causes the mRNA to localize selectively within activated dendritic domains (Figure 8).

With the goal of developing a standard terminology, we propose the following terms to refer to the steps involved in mRNA trafficking and localization within neurons. First, we propose the term "routing" to refer to the processes through which particular mRNAs are retained in the cell body or exported into dendrites or axons. The signals that direct the routing (which in other cell types are often located in the mRNA sequence) would then be termed "routing signals." This signal has also been called an "RNA zip code" (Steward and Singer, 1997). Second, "localization" refers to the selective accumulation of mRNA at a particular intracellular site (for example, a synapse that has recently been activated). Signals within the mRNA sequence or the encoded protein that mediate localization would then be termed "localization signals," and the addresses that mark an intracellular site and cause a particular mRNA to localize selectively would be termed "address markers." Our study provides the first direct evidence that neurons actually do possess mechanisms for both routing and localization and indicates that localization is mediated in part by signals that are generated by synaptic activation.

Routing and Localization of *Arc* mRNA are Mediated by Signals in the mRNA Sequence

Studies of other cell types suggest that routing and localization of mRNA are often mediated by different signals; these signals can in principle lie in either the mRNA or the encoded protein (for a recent review, see Steward and Singer, 1997). In the case of *Arc*, previous studies have demonstrated that the routing of *Arc* mRNA into dendrites continues in the absence of protein synthesis (Wallace et al., 1998), suggesting that selection for dendritic transport is mediated by a routing signal within the mRNA itself. These previous experiments involved systemic injections of cycloheximide, which inhibits protein synthesis by blocking elongation of the nascent peptide chain but does not disaggregate polysomes. Hence, the question remained about whether *Arc* mRNA was transported in a complex with ribosomes. The present study provides additional insights regarding this question by demonstrating that the selective delivery of *Arc* into dendrites also continues in the presence of puromycin, which inhibits protein synthesis by causing premature peptide chain termination, which in turn causes a disaggregation of mRNA and ribosomes (Reid et al., 1970). While it remains to be confirmed that puromycin does cause a disaggregation of *Arc* mRNA and polyribosomes, it seems likely that the *Arc* mRNA that appears in dendrites in the presence of puromycin is not in a complex with ribosomes.

The present study also demonstrates that the selective localization of *Arc* mRNA at active synapses is not disrupted by inhibiting protein synthesis. Hence, neither dendritic transport nor localization can be mediated by a mechanism that depends on the nascent protein. Instead, both routing of *Arc* mRNA into dendrites and localization at active synapses most likely occur based on signals in the mRNA sequence itself.

mRNA Targeting Provides a Potential Mechanism for Enduring Forms of Synapse-Specific Plasticity

The novel mechanism revealed here, through which recently synthesized mRNAs are targeted to active synapses, is ideally suited to mediate protein synthesis–dependent, synapse-specific long-term plasticity. The pattern of stimulation used to induce mRNA localization is the one that is typically used to induce LTP in the dentate gyrus and is preferred because this kind of activity simulates patterns of spike trains seen in cortical neurons (for a discussion, see Bliss and Lynch, 1988). It is important to emphasize, however, that it was not our goal in the present experiments to establish a direct link between the induction and synaptic localization of *Arc* mRNA and the induction of enduring LTP. Establishing this link will require an evaluation of whether the patterns of activation that are necessary and sufficient to induce LTP are also necessary and sufficient to induce mRNA localization. The fact that newly synthesized Arc protein accumulates selectively in the dendritic laminae in which *Arc* mRNA is localized also suggests that targeting of the mRNA underlies a local synthesis of the protein. Nevertheless, to completely exclude the possibility that there are separate mechanisms that target *Arc* mRNA and protein, it will be necessary to develop ways to disrupt mRNA targeting and then evaluate whether this also disrupts protein localization. It remains to be established whether local synthesis of Arc protein plays a key role in synaptic plasticity and whether *Arc* is unique in this regard or is representative of a family of genes that are targeted to synapses by activity.

Experimental Procedures

Neurophysiological Techniques

Adult male Sprague-Dawley rats were anesthetized with Nembutal or urethane and positioned in a stereotaxic apparatus. Stimulating and recording electrodes were positioned stereotaxically so as to selectively activate different afferent projections while recording in the dentate gyrus as described in Levy and Steward (1979) and Steward et al. (1990). To activate the projections from the medial EC, a monopolar stimulating electrode (an insulated tungsten microelectrode) was positioned at 4.0 mm lateral to the midline and 1.0 mm anterior to the transverse sinus. To activate the projections from the lateral EC, the electrode was positioned at 5.5 mm lateral and 1.5 mm anterior to the transverse sinus. The commissural projections to the dentate gyrus were activated by positioning the stimulating electrode in the CA3/hilar region contralateral to the recording electrode (coordinates for the stimulating electrode were 4.0 mm posterior to bregma and 2.0 mm lateral to the midline). In all cases, the depth of the stimulating electrode was adjusted so as to obtain a maximal evoked response in the dentate gyrus at minimal stimulus intensity.

Recording electrodes were glass micropipettes filled with 0.9% saline that were positioned at 3.5 mm posterior to bregma and 1.5–2.0 mm lateral to the midline. The recording electrodes were positioned in the cell layer of the dentate gyrus based on the evoked responses generated by EC stimulation. For the experiments involving stimulation of the commissural pathway, the recording micropipette contained 40 mM bicuculline as described in Steward et al. (1990). The tips of the electrodes were broken off to promote diffusion of the inhibitors into the tissue.

After positioning the stimulating and recording electrodes, stimulus intensity was set so as to evoke an ~1–3 mV population spike. Single test pulses were then delivered at a rate of 1/10 sec for 5–10 min so as to determine baseline response amplitude. Then, trains of high frequency stimuli (8 pulses at 400 Hz) were delivered at a rate of 1/10 sec for 30 min or 1 or 2 hr. At the end of the period of high frequency stimulation, test responses were delivered in order to determine the extent of the synaptic potentiation that had been induced. Control experiments involved delivering the same number and intensity of stimuli over the same time period (2 hr) but at a low frequency that does not cause LTP (0.8 pulses/sec).

In the experiment in which an ECS was elicited prior to perforant path stimulation, stimulation and recording electrodes were positioned, test pulses were collected, and then an electroconvulsive seizure was induced via ear clip electrodes as described in Wallace et al. (1998). Response amplitude was monitored for 10 min, and then high frequency stimulation was initiated as described above.

To locally inhibit protein synthesis within the dentate gyrus, recording micropipette electrodes were filled with either puromycin or cycloheximide (20 mg/ml or 25 mg/ml of 0.9% saline, respectively). The area of effective protein synthesis inhibition was defined by immunostaining sections for Arc protein or for c-fos protein, which are induced by the stimulation. The area of effective protein synthesis inhibition produced in this way was about 1.5 mm in diameter. In two additional experiments, protein synthesis was blocked globally by injecting cycloheximide systemically (20 mg/kg body weight) as described in Wallace et al. (1998).

In Situ Hybridization and Immunocytochemistry

At the termination of the neurophysiological experiment, rats received a lethal dose of anesthetic (100 mg/kg of Nembutal given intraperitoneally). When deeply anesthetized, the animals were perfused with 4% paraformaldehyde. The brains were removed and stored in fixative overnight; on the following day, the brains were sectioned with a vibratome, and sections were collected and stored in phosphate buffer (pH 7.4).

For in situ hybridization, sections were mounted on polylysine-coated microscope slides and then hybridized with digoxygenin-labeled cRNA probes as described in Wallace et al. (1998). Details regarding the cRNA probes used for *Arc*, NGFI-A/*Zif 268*, MAP2, and CaM Kinase II mRNAs have been described previously (Paradies and Steward, 1997; Wallace et al., 1998).

For immunocytochemistry, vibratome sections were heat-treated (95°C for 5 min) to recover antigenicity and then immunostained by using an antiserum for Arc protein (for details on antibody production and characterization, see Lyford et al., 1995). Sections were incubated for 72 hr in the primary antibody (1:200 dilution); subsequent immunocytochemical procedures were as described in Wallace et al. (1998).

For quantitative assessment of labeling, OD measurements were taken across the granule cell layer and molecular layer with an M4 Microcomputer Imaging Device (MCID), Imaging Research. Digital images of an area of the suprapyramidal blade of the dentate gyrus were collected at 400× (approximately the area illustrated in Figure 1G). The light intensity was adjusted so that areas exhibiting background levels of labeling (the hilus contralateral to the side of the stimulation) were just above threshold, whereas areas exhibiting maximal levels of labeling (the granule cell layer ipsilateral to the stimulation) were within the measuring range. Then, a series of OD measurements were taken across the granule cell layer and molecular layer with a 20 μm × 20 μm measuring frame (see Figure 1). A row of five separate measurements were taken at each level of the granule cell layer and molecular layer, and the OD values at each level (called row numbers in the figures) were averaged. The values in the graphs illustrate the mean and standard deviation of the five measurements.

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